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Stress impairs acquisition of delay eyeblink conditioning in men and women

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ABSTRACT

In rodents stress impairs delay as well as trace eyelid conditioning in females, but enhances it in males. The present study tested the effects of acute psychosocial stress exposure on classical delay eyeblink conditioning in healthy men and women. In a between subject design, participants were exposed to psychosocial stress using the Trier Social Stress Test (TSST) or a control condition which was followed by a delay eyeblink classical conditioning procedure. Stress exposure led to a significant increase in salivary cortisol and impaired acquisition of conditioned eyeblink responses (CRs). This was evident by a later first CR and an overall lower CR rate of the stress group. The stress-induced acquisition impairment was observed in both women and men. Subjects failing to show a stress-induced cortisol increase (cortisol non-responder) were not impaired in acquisition. Our findings indicate that acute stress, possibly via activation of the hypothalamus–pituitary–adrenal (HPA) axis, reduces the ability to acquire a simple conditioned motor response in humans.

1. Introduction

Stress and its associated activation of the hypothalamus–pituitary–adrenal (HPA) axis is known to influence learning and memory. Glucocorticoids released from the adrenal cortex are important neuroendocrine mediators in this regard. Stress can enhance or impair memory depending on a number of modulatory variables (Conrad, 2005; Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Joels, Pu, Wiegert, Oitzl, & Krugers, 2006). Effects of stress have been investigated on different forms of memory (Lupien, Maheu, Tu, Fiocco, & Schramek, 2007; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Wolf, 2008).

In human subjects, stress effects have so far been established for fear conditioning which is mediated by the amygdala (LaBar & Cabeza, 2006) and for trace eyeblink conditioning which involves hippocampal mechanisms (Cheng, Disterhoft, Power, Ellis, & Desmond, 2008; Christian & Thompson, 2003; Woodruff-Pak & Disterhoft, 2008). Stress induced by the cold pressor test led to enhanced trace eyeblink conditioning in healthy men (Duncko, Cornwell, Cui, Merikangas, & Grillon, 2007), while cortisol administration was found to impair trace conditioning in patients with post-traumatic stress disorder (Vythilingam et al., 2006). Moreover, Morbus Cushing patients, who are characterized by substantially elevated endogenous cortisol levels, showed impaired trace conditioning (Grillon, Smith, Haynos, & Nieman, 2004). Stress-induced cortisol elevations or basal cortisol levels were associated with enhanced fear conditioning, especially in men (Jackson, Payne, Nadel, & Jacobs, 2006; Zorawski, Blanding, Kuhn, & LaBar, 2006; Zorawski, Cook, Kuhn, & LaBar, 2005), while pharmacologically induced elevated cortisol levels lead to impaired fear conditioning in men (Stark et al., 2006).

Thus,while stress effects have been established for fear conditioning and trace eyeblink in humans, there is as yet no evidence for stress effects on simple delay eyeblink conditioning which have been comprehensively studied in rodents. Delay eyelid conditioning is one of the most widely used conditioning procedures across species (Christian & Thompson, 2003). Stress effects on delay (as well as trace) conditioning in rats were modulated by sex. While male animals showed poorer acquisition under resting condition their performance was enhanced by stress. The opposite pattern was observed in females. Here performance was good under rest but was impaired by stress (Shors, 2004).

Acquisition of delay eyeblink conditioning is dependent upon the functional integrity of cerebellar circuits, while hippocampal damage does not affect CR acquisition or retention (Christian & Thompson, 2003; Daum et al., 1993a; Schugens & Daum, 1999). However, while the hippocampus is not necessary for delay eyelid conditioning per se, pharmacological disruption of normal hippocampal function may adversely affect acquisition (Solomon, Solomon, Schaaf, & Perry, 1983), and hippocampal mechanisms may be critical for the modulatory effects of stress on conditioning in rodents (Bangasser & Shors, 2007).

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The present study aimed to assess the effects of psychosocial stress on delay eyeblink conditioning in humans and its potential modulation by sex. Based on the data from rodent studies, acute stress was expected to impair acquisition in women and to enhance acquisition in men.

2. Materials and methods

2.1. Participants

A total of 67 young healthy participants were recruited. For the main analysis, only subjects who showed a cortisol increase in response to the stressor (+30 min minus baseline > 0) were included into the stress group. This strategy was chosen in order to ensure a robust HPA response in the stress group and in order to avoid the possibility that the known sex differences in the response to this kind of stressor (Kajantie & Phillips, 2006; Kudielka & Kirschbaum, 2005; Stroud, Salovey, & Epel, 2002) might bias the conditioning results. Fourteen subjects (10 women) from the stress group did not fulfill this criterion and thus were excluded from the initial analysis; however an additional analysis focusing specifically on the non-responder group is presented at the end of the results section. The initial data set thus consisted of 53 healthy, non-smoking subjects (27 female) between 20 and 35 years of age (mean 24.3 years, *SD* = 3.3). The average body mass index (BMI = weight in kg/height in m^2) of the subjects was 22.2 (range: 18–27; *SD* = 2.3). None of the participants was taking regular medication or had a history of any psychiatric or neurological treatment.

None of the female subjects were using hormonal contraceptives and conditioning was assessed in the ovulatory phase (13th–15th day of a regular cycle between 26 and 32 days) in all women. This phase was chosen because of high estradiol levels during this cycle phase which have been shown to be a prerequisite for the stress-induced conditioning impairment in female rodents (Wood & Shors, 1998).

Twenty-four subjects (13 female) were allocated to the stress condition; the remaining subjects completed the control condition (see below). The four groups did not differ significantly in age or BMI (see Table 1). The study was performed in accordance with ethical standards laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Medical Faculty of the Ruhr-University, Bochum, Germany. Written informed consent was obtained from all subjects.

2.2. Procedure

Participants arrived between 9:00 AM and 12:00 PM and filled out a mood questionnaire (positive and negative affect schedule; PANAS, (Watson, Clark, & Tellegen, 1988). The PANAS entails 10 items assessing positive affects (e.g. interested and enthusiastic) and 10 items assessing negative affects (e.g. upset and ashamed), each item is rated on a 5-point scale.

Next participants were either exposed to a psychosocial laboratory stressor (Trier Social Stress Test; TSST, see below) or a standardized control condition, respectively. Saliva was collected before, immediately after, 10 and 30 min after the TSST or the control task. The mood questionnaire was also completed again after stress induction or the control task. Ten minutes after completion of the stress test, at a time when cortisol levels reach their peak (Kuhlmann, Piel, & Wolf, 2005), participants underwent a delay eyeblink conditioning task (60 acquisition and 10 extinction trials), followed by a post-experimental interview.

Table 1
Demographic data, body mass index (BMI), Unconditioned Stimulus (US) Intensity rating and Unconditioned Response (UR) amplitude in the stress and control group.

	Stress	Controls
Age (years)	25.1 (*SD* = 3.3)	23.6 (*SD* = 3.2)
Sex	13 female, 11 male	14 female, 15 male
BMI (kg/m^2)	22.2 (*SD* = 2.3)	22.2 (*SD* = 2.4)
US rating	2.8 (*SD* = 0.6)	2.7 (*SD* = 0.5)
UCR amplitude (A/D)	2596.7 (*SD* = 1339.4)	3309.9 (*SD* = 1745.5)

A/D units = analog-to-digital converter units, see Daum et al. (1993a, 1993b).

2.3. Stress induction

The TSST is a well-established procedure to induce a HPA response in the laboratory (Dickerson & Kemeny, 2004). It consists of a short preparation period (2 min) followed by free speech (5 min) representing a job interview in front of a committee (consisting of one male and one female experimenter) and a mental arithmetic task lasting another 5 min (counting backwards from 2043 in steps of 17). The performance of the participants is recorded on videotape (Kirschbaum, Pirke, & Hellhammer, 1993). The non-stressful control condition is similar in physical and mental demands (free speech and mental arithmetic task, the subject being alone in a room), while it lacks the stress-inducing components of the TSST (such as socio-evaluative threat; for further details, see Kuhlmann et al., 2005).

2.4. Cortisol assessment

Saliva was collected using Salivette collection devices (Sarstedt, Nuembrecht, Germany). Free salivary cortisol levels were measured using an immunoassay (IBL, Hamburg, Germany). Inter- and intra-assay variations were below 15%.

2.5. Conditioning schedule

Ten minutes after stress induction, a delay eyeblink conditioning procedure was administered which has previously yielded acquisition impairments after cerebellar, but not after hippocampal damage in human subjects (Daum et al., 1993b). Acquisition comprised 6 blocks of 10 trials and each block consisted of 7 reinforced and 3 unreinforced trials in random order. On a reinforced trial, an 800-ms conditioned stimulus (CS) tone (1000 Hz, 70 dB) was followed after 720 ms by an 80-ms airpuff unconditioned stimulus (US), both stimuli coterminated. The CS tone was presented binaurally via headphones, the airpuff US (200 mm Hg) was administered to the cornea of the right eye from a distance of 1 cm via a nozzle mounted on goggles. Acquisition was followed by 10 extinction trials without any warning of the subjects. Intertrial intervals ranged from 10 to 14 s with a mean of 12 s. The technical details of stimulus presentation are described in detail elsewhere (Daum et al., 1993b). Airpuffs were administered via an electrically operated air valve mounted on the goggles, and eyelid movements were recorded with a photocell system attached to the goggles.

Subjects were seated in a sound-attenuating and electrically shielded room, facing a computer monitor at a distance of 80 cm. They were instructed to fixate the center of the screen and to avoid deliberate closure of their eyes and body movements. They were instructed that their memory for the session would be tested and that they would hear some tones and occasionally feel an airpuff to the eye which would neither be harmful or painful.

Conditioning was followed by a post-experimental interview (see Bellebaum & Daum, 2004) to assess whether subjects had insight into the stimulus contingencies during acquisition (“What was the experiment about?”, “Did the airpuff come after every tone?”) and extinction phase (“What had happened at the end of the experiment?”). A subject was rated as aware if she or he indi-

cated that the tone predicted the administration of an airpuff in more than 50% of the trials. In addition a subjective rating of US intensity was obtained.

2.6. Eyelid conditioning data analysis

Eyeblink data were analyzed offline using the EEG Analyst software (Daum et al., 1993b). Eyelid movements were sampled at 200 Hz for 2.56 s, starting 580 ms before CS onset up to an interval of 1.120 ms after offset of both stimuli. Onset and peak amplitude markers were applied to a blink if the change in the curve exceeded an amplitude of 25 A/D units for 25 ms (slope criterion) and if the peak amplitude exceeded 75 A/D units (i.e. approximately 0.8 mm of eyelid movement). A conditioned response (CR) was defined as a closing movement of the eyelid with a latency between 450 ms after tone onset and US onset. The trial number of first CR and CR frequency in each acquisition block and extinction were determined for each subject. Eyelid closures with latencies between US impact and 160 ms after US onset were defined as URs. UR amplitude was analyzed for the first 10 trials only because of a blend of CRs and URs in later acquisition stages.

3. Results

3.1. Cortisol level

The cortisol stress responses for female and male subjects are illustrated in Fig. 1. A repeated-measures ANOVA with factors Group (stress versus control), Sex (female versus male) and Time (baseline, +1, +10 and +30 min) yielded a significant Group × Time interaction ($F_{3,147} = 18.48$, $p < .001$). Subsequent paired comparisons revealed significantly higher cortisol levels at 10 min ($t_{51} = 3.09$, $p = .002$) and 30 min ($t_{51} = 3.00$, $p = .004$) after stress compared with the control condition. Stress and control group did not differ with regard to pre-stress cortisol level (baseline). Neither the main effect of Sex nor any of the other interactions were significant.

3.2. Mood questionnaire

Data from the PANAS were analyzed with a repeated-measures ANOVA with factors Group (stress versus control), Sex (female versus male), and Time (before and after treatment) for both scales separately. Analysis of negative affect yielded a significant Group - Time interaction ($F_{1,49} = 9.8$, $p = .003$). Stress led to an increase in negative affect (baseline: $M = 1.4$. $SD = 0.47$; post-stress: $M = 1.7$, $SD = 0.62$), while there was no significant change in the control condition (baseline: $M = 1.26$, $SD = 0.42$; post-control: $M = 1.1$, $SD = 0.28$). No other main effect or interaction was significant. Analysis of positive affect did not yield any significant stress effects.

3.3. Delay conditioning

3.3.1. UCR amplitude

Means and SDs for the median UCR amplitude and the subjective ratings of US intensity are presented in Table 1. ANOVAs did neither reveal significant effects for the factor Group nor a significant Group by Sex interaction for these variables.

3.3.2. First CR

The mean trial number of first CR as a measure of conditioning onset is depicted in Fig. 2 for each group. ANOVA with factors Group (stress versus controls) and Sex yielded a main effect of Group ($F_{1,49} = 6.7$, $p = .012$), indicating earlier CRs in the control ($M = 6.0$, $SD = 4.3$) compared to the stress group ($M = 10.3$, $SD = 7.4$). None of the other comparisons reached significance.

3.3.3. CR frequency

The development of CR frequency across the six acquisition blocks and the extinction block is illustrated in Fig. 3. For acquisition, repeated-measures ANOVA with factors Group (stress versus controls), Sex (females versus males) and Block (block 1–6) yielded significant main effects of Block (linear trend: $F_{1,49} = 45.01$, $p < .001$) and Group ($F_{1,49} = 11.6$, $p = .001$), with significantly higher average CR rates in the control compared to the stress group. The linear trend represents significant learning across blocks (see Fig. 3). The Group by Block interaction yielded a trend ($p < .10$). None of the other comparisons reached significance.

3.3.4. Extinction

Repeated-measures ANOVA with the factors Block (last acquisition block versus extinction block), Group (stress versus control) and Sex (female versus male) yielded a main effect of Block ($F_{1,49} = 46.8$, $p < .001$), indicating extinction, and a main effect of Group ($F_{1,49} = 6.7$, $p < .012$) with higher CR rates in the control compared to the stress group (see Fig. 3). None of the interactions reached significance.

3.4. Awareness

Five subjects could not be clearly allocated to the aware and unaware subgroups on the basis of their ratings. Seventeen of 21

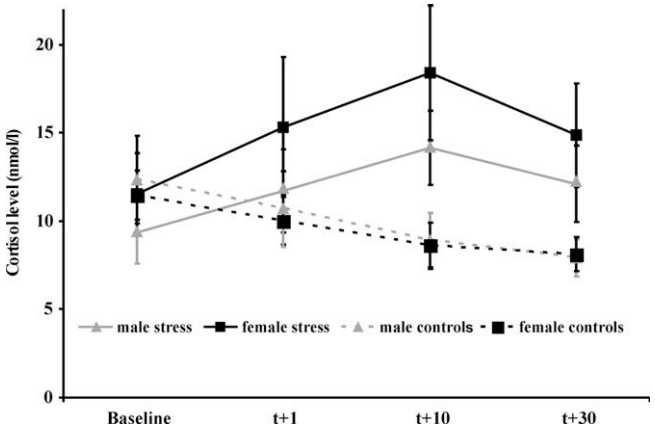

Fig. 1. Cortisol levels before (baseline) and after (+1, +10, and +30 min) exposure to a laboratory stressor or a control condition. Stressed subjects of both sexes displayed significantly higher cortisol levels 10 and 30 min after cessation of the stressor. During this interval delay eyelid conditioning took place.

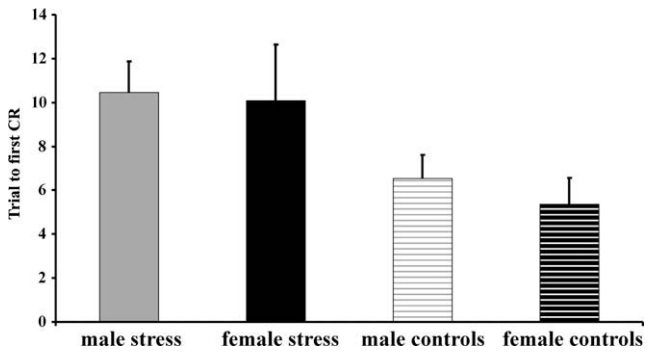

Fig. 2. Effects of stress on trials needed to show a first conditioned response. Stressed men and women needed significantly more trials before they showed the first CR.

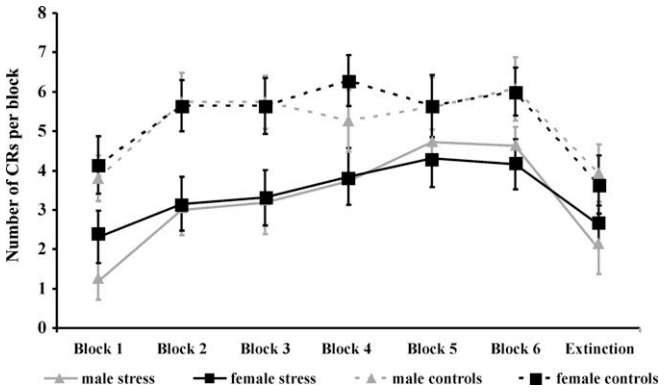

Fig. 3. Effects of stress on acquisition and extinction of a delay eyeblink response. Stress lead to a slower acquisition in men and women but had no effect on extinction.

subjects (9 women, 8 men) of the stress group and 24 of 27 subjects of the control group (12 women, 12 men) were rated as aware. Chi-square analysis revealed that the proportion of aware subjects did not differ significantly between the two groups ($p > .20$).

3.5. Stress-induced conditioning impairments: exploring the role of the stress-induced cortisol increase

In order to ensure a robust cortisol response in the stress group and in order to ascertain a similar response in both sexes, those subjects ($n = 14$) not showing a cortisol increase in response to the stressor were initially excluded (see above). However, a closer look at this group is of interest, since the data may elucidate whether or not a stress-induced HPA activation is required for the observed learning deficits.

We therefore conducted an additional analysis with three groups (controls, stress responders, and stress non-responders). Due to the small number of men in the non-responder group ($n = 4$) the factor sex could not be included in this analysis. As illustrated in Fig. 4, the non-responder group showed a highly similar conditioning pattern to the control group. An ANOVA with the factor group (three levels) revealed a significant effect for first CR as well total CRs. Bonferroni-adjusted post-hoc tests revealed that the non-responder group differed significantly ($p < .05$) from the responder group in the number of trials needed to show a first CR (responder group: $M = 10.3$, $SD = 7.4$; non-responder group: $M = 5.3$, $SD = 5.5$) as well as in the total number of CRs (responder group: $M = 23.1$, $SD = 12.5$; non-responder group: $M = 37.1$, $SD = 17.7$).

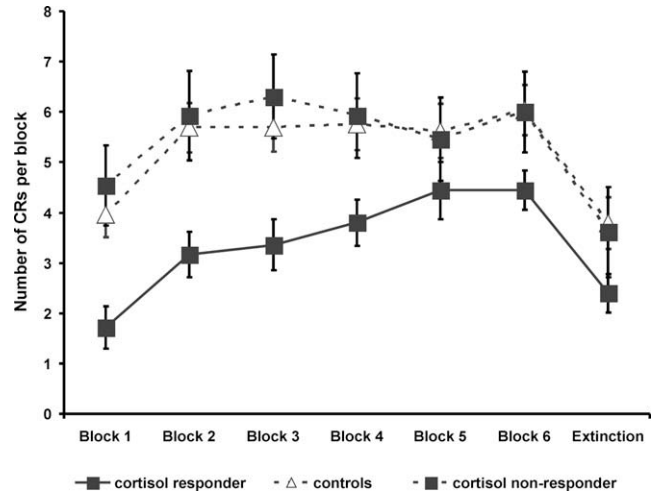

Fig. 4. Influence of cortisol responder status on the effects of stress on acquisition of a delay eyeblink response. Subjects not showing a cortisol increase in response to stress exposure ($n = 14$) acquired the delay eyeblink response highly similar to non-stressed controls ($n = 29$). Both groups were significantly better than those subjects showing a cortisol response to the stressor ($n = 24$).

A second approach to investigate the relevance of cortisol in mediating the observed effects was done using correlations. For this analysis the area under the cortisol curve (AUC) was calculated in order to obtain a single integrative value for cortisol (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). For the analysis with all subjects ($n = 67$) there was a significant positive correlation between the cortisol AUC and the first CR ($r = .24$, $p < .05$). In addition there was a significant negative correlation between the cortisol AUC and CR rate during acquisition ($r = -0.25$, $p < .05$). Thus higher cortisol levels were associated with a later first CR and a reduced overall number of CRs.

4. Discussion

This study aimed to assess the effects of acute stress on delay eyeblink conditioning in men and women. As expected, psychosocial stress caused a rise in salivary cortisol, accompanied by an increase in negative mood. Acquisition of delay eyeblink conditioning was impaired in the stress group compared to a control condition in both men and women. Stress led to a later occurrence of the first CR and an overall lower CR frequency across acquisition and during extinction, with both the stress and control groups showing significant learning. While stressed participants showed increased CR rates over the course of the 6 blocks, they did not reach the level achieved by the control group. In contrast to its effect on acquisition, stress had no selective effect on extinction. Since the stress and the control group did not differ significantly on UR amplitude or subjective US intensity ratings, the stress effects on acquisition cannot simply be attributed to altered processing of the airpuff US.

The stress paradigm used in our study (the TSST) has repeatedly yielded stronger cortisol increases in men compared to women (Kajantie & Phillips, 2006; Kudielka & Kirschbaum, 2005). To ensure a robust HPA stress response in the present study, we only included women who did not use hormonal birth control which has been linked to a reduced free cortisol stress response (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). In addition we excluded non-responders, i.e. participants with an absent cortisol response from the initial analysis. This strategy led to a comparable cortisol response in both sexes.

Awareness of stimulus contingencies may affect acquisition of eyeblink conditioning in young healthy subjects (Bellebaum & Daum, 2004; Clark, Manns, & Squire, 2002; Clark & Squire, 1998), but since the proportion of aware and unaware subjects did not differ between the stress and control groups, it is unlikely that awareness contributed significantly to the observed stress effects on acquisition.

In addition to the overall stress effect on acquisition, the current results yielded support for the notion that the stress-induced activation of the HPA axis underlies the observed effects. The small group of subjects not showing a cortisol increase in response to the stressor (cortisol non-responder) showed a conditioning performance which was highly similar to the control group and significantly different from the cortisol responder group. Thus stress exposure only caused a conditioning impairment if subjects responded with a cortisol increase to the stressor. Similar observations have been made in other human studies looking at working

memory or delayed memory retrieval (Buchanan, Tranel, & Adolphs, 2006; Elzinga & Roelofs, 2005). In addition, correlation analysis revealed that a larger cortisol area under the curve was associated with a later first CR and a lower number of total CRs. Thus the unimpaired conditioning of the non-responders and the significant correlations suggest that an activation of the HPA axis is a prerequisite for the occurrence of the stress-induced conditioning impairment. Having said this it has to be emphasized that these correlational observations should not be interpreted in a causal fashion. Since multiple neuroendocrine as well as psychological changes occur in response to stress the effects observed in our present study could also be caused by other, non-HPA related, mechanisms. Ultimately pharmacological studies are needed to test the role of cortisol as a mediator in the stress-induced impairments observed in this study.

In a number of conditioning studies, Shors and co-workers had established a pattern of differential stress effects on eyeblink conditioning in rodents, with acquisition impairments in females and enhanced acquisition in males (Shors, 2004; Shors, Beylin, Wood, & Gould, 2000). While our results in women match the rodent data, our findings of a comparable stress-induced learning deficit in men are inconsistent with these findings. There are a number of possible explanations for this. In rodents, male animals generally learn slower than females, leading to significant differences in the no-stress condition (Shors, 2004), a sex difference which was not observed in the current study where acquisition in the control condition was comparable for men and women. The sex differences in rodents in the no-stress condition might suggest sex differences in learning mechanisms which in turn might be differentially susceptible to stress. In support of this notion is the finding that in a spatial memory task with male superiority in the no-stress condition acute stress leads to impaired performance in males but to enhanced performance in females (Conrad et al., 2004).

It is of course also possible that differences in task difficulty and/or in task-induced arousal (Conrad, 2005; Diamond et al., 2007; Sandi & Pinelo-Nava, 2007) might underlie the different result patterns in humans versus rats. Finally the used stressors (tail shock or swim stress in rodents, psychosocial paradigms in humans) are also considerably different from each other. Thus more research efforts are needed to characterize the specific emotional and cognitive processes which are modulated by stress in a sex-dependent fashion (Cahill, 2006; Wolf, 2008).

Studies of stress effects on human eyeblink conditioning are sparse which is in contrast to the well-documented effects on episodic memory (Lupien et al., 2007; Wolf, 2008). For trace conditioning, a more moderate physiological stressor (the cold pressor stressor) was previously found to enhance trace eyeblink conditioning in a sample of young men (Duncko et al., 2007). This stressor is substantially shorter than the TSST and failed to induce a significant HPA response. Nonetheless, increased activation of the sympathetic nervous system induced by a mild stressor might enhance (trace) eyelid conditioning, while a more robust cortisol increase induced by a psychosocial laboratory stressor could causes a delay acquisition impairment. This idea is supported by recent findings of impaired trace eyeblink conditioning after pharmacological cortisol administration or in endogenous hypercortisolemia (Grillon et al., 2004; Vythilingam et al., 2006).

In fear conditioning, sex differences of stress effects are frequently observed, with evidence of stress-enhanced conditioning in male participants (Jackson et al., 2006; Stark et al., 2006; Zorawski et al.,2005, 2006), but these effects are not directly comparable to eyeblink conditioning because of the differences in the underlying neuronal circuitry of eyeblink and fear conditioning (Christian & Thompson, 2003; LaBar & Cabeza, 2006; Woodruff-Pak & Disterhoft, 2008). The present findings indirectly add to the accumulating evidence of differential stress effects on cognitive processes mediated by the amygdala versus the hippocampus (Conrad, 2005; Diamond et al., 2007; Sandi & Pinelo-Nava, 2007).

Acquisition of delay eyeblink conditioning is not dependent upon the functional integrity of the hippocampus (Cheng et al., 2008; Christian & Thompson, 2003; Clark, Manns, & Squire, 2001; Daum, Channon, & Gray, 1992). However, in rodents, hippocampal mechanisms mediate the stress effects on acquisition of eyeblink conditioning (Bangasser & Shors, 2007). This is consistent with earlier findings suggesting that pharmacological manipulation of hippocampal function using an anticholinergic drug had more detrimental effects on delay eyeblink conditioning than hippocampal lesions (Solomon et al., 1983). Similarly beneficial effects of pharmacological treatments on delay conditioning in older rabbits were mediated by hippocampal mechanisms (Woodruff-Pak, Li, Hinchliffe, & Port, 1997).

Electrophysiological evidence indicate that the stress-associated activation of the sympathetic nervous system and the HPA axis initially lead to a brief excitation of the hippocampus, followed by an inhibition caused by increased glucocorticoid signalling (Diamond et al., 2007; Joels et al., 2006). This pattern is in line with human functional neuroimaging studies showing reduced hippocampal activity after cortisol administration (de Leon et al., 1997; de Quervain et al., 2003; Oei et al., 2007). Future experiments are needed in order to characterize the temporal dynamics of the acute stress effects on human eyelid conditioning. Moreover in rodents the effects of stress on eyeblink conditioning persists for at least 24 h (Shors, 2004). It awaits to be shown whether such persistent effects can be detected in humans as well.

In sum the present findings indicate that exposure to an acute psychosocial stressor leads to impaired acquisition of delay eyeblink conditioning in men and women. We hypothesize that the observed impairment reflects an inhibitory influence of cortisol on the hippocampus which affects the basic cerebellar–brainstem circuits mediating the conditioned response. However, direct effects of stress on the cerebellum are also conceivable. Future human neuroimaging studies will allow to disentangle the neuroanatomical regions involved in this acute stress effect.

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